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PapD chaperone function in pilus biogenesis depends on oxidant and chaperone-like activities of DsbA

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ABSTRACT **Adhesive P pili of uropathogenic *Escherichia coli* were not assembled by a strain that lacks the periplasmic disulfide isomerase DsbA. This defect was mostly attributed to the immunoglobulin-like pilus chaperone PapD, which possesses an unusual intrasheet disulfide bond between the last two β-strands of its CD4-like carboxyl-terminal domain. The DsbA-dependent formation of this disulfide bond was critical for PapD's proper folding *in vivo*. Interestingly, the absence of the disulfide bond did not prevent PapD from folding *in vitro* or from forming a complex with the pilus adhesin *in vitro*. We suggest that DsbA maintains nascently translocated PapD in a folding-competent conformation prior to catalyzing disulfide bond formation, acting both as an oxidant and in a chaperone-like fashion. Disulfide bond formation in pilus subunits was also mediated by DsbA even in the absence of PapD. However, the ability of pilus subunits to achieve native-like conformations *in vivo* depended on PapD. These results suggest that a productive folding pathway for subunits requires sequential interactions with DsbA and the PapD chaperone.**

P pili are adhesive fibers assembled by uropathogenic *Escherichia coli* and are representative of a large family of pili produced by Gram-negative bacteria (1). Pilus biogenesis requires the periplasmic immunoglobulin-like PapD chaperone and the PapC outer membrane assembly protein (2, 3). P pili are composite fibers. The pilus rod consists of PapA subunits that are arranged in a cylindrical rod (35). Joined to the distal ends of each pilus rod is a thin fibrillum composed of four specialized subunit types—PapK, PapE, PapF, and the adhesion PapG—arranged in an open helical architecture (4, 5). Genes important in pili biogenesis, *papA–K*, are linked in an operon and their expression is coordinately controlled (1). The synthesis of P pili occurs via the following pathway. The subunits are exported across the cytoplasmic membrane and their signal peptide is removed. PapD binds to each subunit, forming periplasmic chaperone–subunit complexes that are targeted to the outer membrane assembly site consisting of the PapC usher. At the assembly site, the complex dissociates allowing incorporation of subunits into the pilus fiber across the outer membrane, in a specific order determined by complementary surfaces on each subunit type (5). In the absence of an interaction with PapD, the pilus subunits aggregate nonproductively and are proteolytically degraded (H. Jones and S.J.H., unpublished work). PapD–PapG, PapD–PapA, PapD–PapE, and PapD–PapK chaperone–subunit preassembly complexes have been isolated from periplasmic extracts and characterized (6, 7) and the PapD–PapA complex was shown to represent a true *in vivo* intermediate in pilus assembly (7). The subunit moieties in the chaperone–subunit complexes exist in native-like states as demonstrated by the presence of disulfide bonds and secondary structure in the adhesin and by the ability of the adhesin to bind to its receptor (6). However, it is not known whether subunit folding is chaperone assisted *in vivo*.

The folding of presecretory proteins in the cytoplasm is usually prevented by interactions with chaperones and slowed by the interference of their signal peptide (8–10). It was reported that disulfide bond formation of a secretory protein in the cytoplasm was prevented by thioredoxin reductase (11) and that the disulfide isomerase DsbA was involved in accelerating the rate of disulfide bond formation in the periplasmic space (12, 13). In the present work the role of DsbA in the assembly of *E. coli* P and type 1 pili was investigated. Type 1 pili are also composite fibers produced by all members of the *Enterobacteriaceae* family (H. Jones and S.J.H., unpublished work) and require the FimC chaperone for their assembly. In contrast to PapD, FimC does not contain any cysteines. However, all of the P and type 1 pilus subunits possess an intramolecular disulfide bond between two cysteines spaced ≈30 residues apart that are characteristic of almost all pilus subunits (14). The PapG and FimH adhesins of P and type 1 pili, respectively, are about twice the molecular weight of the pilins and contain four cysteines that are involved in intramolecular disulfide bonds (6). Since pilus subunits transit through the periplasmic space, we sought to determine the role of DsbA in chaperone–subunit complex formation and pilus assembly.

MATERIALS AND METHODS

Strains and Plasmids. *E. coli* MC1061 was used as a recipient for cloning. JCB570 and JCB571 (*dsbA::kan*) are two isogenic strains kindly provided by Jon Beckwith and Jim Bardwell (Harvard Medical School, Boston). The following primers were used to introduce mutations in *papD* cloned in bacteriophage vector M13mp19: C207S (Cys^{207} → Ser), 5′-GCTACCATTAGAGATAAAGAC-3′; C212S, 5′-CTCTTTTTTCACAGAGGAACGGCTAC-3′; and C212Stop, 5′-CTCTTTTTTCACAGATCAACGGCTAC-3′. The double *papD* mutants C207S+C212S or C207S+C212Stop were obtained with the Bio-Rad *in vitro* mutagenesis kit. They were sequenced entirely and recloned as *Eco*RI–*Bam*HI fragments in plasmid pMMB91, resulting in pFJ33 (C207S+C212stop) and pFJ34 (C207S+C212S). pFJ8 (*papD papF*) and pFJ18 (*papD papK*) were obtained by cloning *papD* on an *Eco*RI–*Bam*HI fragment from pLS101 (15) into pFJ7 and pFJ11, respectively (5). pJP2 was obtained by cloning the *Eco*RI–*Bam*HI fragment of pJP1 that contains *papDpapG* (3) into the polylinker of pTrc99A (Pharmacia). pHJ6, pHJ2, and pHJ8 contain, respectively, *papD*, *papA*, and *papG* under the control of the *tac* promoter in pMMB66, and pHJ9203 contains *papD* under the control of the arabinose promoter in a derivative of pBR322 (H. Jones and S.J.H., unpublished

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Abbreviation: MBP–G140, chimeric protein with the 140 carboxyl-terminal amino acids of PapG fused to maltose-binding protein.

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work). pPAP5, pPAP37, pRS2A, pLS101, pLS201, and pJP3 were described previously (7, 15–17).

Native Polyacrylamide Gel Electrophoresis (PAGE). Periplasmic extracts (15) were analyzed by acidic native PAGE (7) and transferred to poly(vinylidene difluoride) for immunoblotting. A 7.5% PAGE system was also used with a 3-(*N*-morpholino)propanesulfonic acid/histidine buffer system at pH 6.5. PapD migrated toward the cathode.

Alkylation of PapD. Wild-type PapD (≈1.5 mg/ml) dialyzed in 20 mM Tris·HCl (pH 8) was denatured by a short incubation with 6 M guanidinium chloride at room temperature, reduced with 10 mM dithiothreitol for 30 min on ice, and incubated for 2 hr on ice in the presence of 50 mM iodoacetic acid. The denaturant and reagents were removed by dialysis against phosphate-buffered saline (120 mM NaCl/2.7 mM KCl/10 mM phosphate buffer salts, pH 7.4; Sigma).

Subunit Binding Activity of PapD *in Vitro*. Samples (10 pmol) of a chimeric protein containing the 140 carboxyl-terminal amino acids of PapG fused to the carboxyl terminus of the periplasmic maltose-binding protein (MBP–G140) were used to coat the wells of a microtiter plate (Z.X. and S.J.H., unpublished work). After an incubation with serial dilutions of native or alkylated PapD, bound PapD was quantitated by an ELISA, and the amount bound to MBP alone was subtracted from the respective values obtained for the binding to the fusion protein. Isoelectrofocusing on precast pH 3–9 gels (Phast system, Pharmacia) was performed after native or alkylated PapD and MBP–G140 (each at ≈0.2 mg/ml) were incubated together for 10 min at 22°C.

Refolding of PapD *in Vitro*. PapD [1 mg/ml in 20 mM potassium 2-(*N*-morpholino)ethanesulfonate (pH 6.5)] was denatured and reduced by a short incubation in 4.8 M urea/20 mM dithiothreitol. The denaturant (urea) concentration was then lowered in a stepwise fashion by dilution in the same buffer. The fluorescence emission of PapD in urea concentrations between 5 and 0.5 M was recorded at 20°C at 325 nm (Photon Technology International fluorometer) after excitation at 290 nm. The final concentration of PapD in the cuvette was 0.1 mg/ml.

Pulse–Chase Analysis and Immunoprecipitation. These procedures have been described (18). The aliquots withdrawn at various times of the chase were immediately mixed with an equal volume of ice-cold 100 mM sodium phosphate, pH 8/100 mM iodoacetamide. Periplasmic extracts in 1 mM iodoacetamide were subjected to immunoprecipitation (18) using the indicated antisera and analyzed by SDS/PAGE and autoradiography. In proteolysis experiments, periplasmic extracts containing labeled PapD were incubated with trypsin (100 μg/ml) at 37°C for 20 min. The digestion was terminated with 1 mM *p*-amidinophenylmethylsulfonyl fluoride. Immunoprecipitated PapD was analyzed by SDS/PAGE and autoradiography.

Other Methods. The preparation of pili, the determination of hemagglutination titers, and pilus ELISA were as described (5, 17).

RESULTS

Type 1 but Not P Pili Are Assembled in a *dsbA*⁻ Background. The role of the periplasmic disulfide isomerase in the assembly of P pili was investigated by measuring the amount of pili produced by the isogenic *E. coli* strains JCB570 (*dsbA*⁺) and JCB571 (*dsbA::kan*) transformed with pPAP5, which encodes the entire *pap* operon. JCB571/pPAP5 cells were reproducibly hemagglutination-negative (titer < 2), in contrast to JCB570/pPAP5 cells, which had a titer of 64. In addition, with anti-P pilus antiserum in an ELISA, no pili were detected in JCB571/pPAP5 pili extracts (titer < 1), in contrast to JCB570/pPAP5 extracts, which had a titer of ≈300.

Interestingly, *dsbA*⁻ cells transformed with a plasmid encoding the entire *E. coli* type 1 operon (pJP3) were mannose-sensitive hemagglutination-positive, signifying their ability to produce type 1 pili, but they had a lower titer than their wild-type isogenic parent (16 for JCB571/pJP3 compared with 128 for JCB570/pJP3). All type 1 and Pap subunits possess an intramolecular disulfide bond between two conserved cysteines. PapD possesses an unusual intrasheet disulfide bond between the last two β-strands of its CD4-like carboxyl-terminal domain in contrast to the type 1 pilus chaperone, FimC, which does not contain cysteines; this difference may explain the ability of *dsbA*⁻ cells to assemble type 1 but not P pili.

This hypothesis was tested by investigating chaperone–subunit complex formation *in vivo*. PapD was coexpressed with either PapF, PapK, PapA, or PapG in the *dsbA*⁺ and *dsbA*⁻ strains using plasmids pFJ8 (*papDpapF*), pFJ18 (*papDpapK*), pHJ9203 (*papD*) and pHJ2 (*papA*), or pHJ9203 (*papD*) and pHJ8 (*papG*), respectively. PapD was also expressed alone in both strains from pHJ6 (*papD*). Chaperone–subunit complexes in periplasmic extracts were detected with appropriate antisera in Western blots of acidic native polyacrylamide gels. PapD–PapG, PapD–PapA, and PapD–PapK chaperone–subunit complexes were detected in periplasmic extracts of JCB570 (*dsbA*⁺) containing the appropriate plasmids (Fig. 1, lanes 1, 3, and 5), whereas little or none of these complexes was found in the isogenic JCB571 (*dsbA*⁻) cells (lanes 2, 4, and 6). Pilus subunits are proteolytically degraded in the absence of an interaction with the chaperone (15). Western blotting of the periplasmic extracts after SDS/PAGE revealed only small amounts of PapA, very little PapG, and no PapF or PapK in *dsbA*⁻ periplasmic extracts, but all these Pap proteins were abundant in dsbA⁺ extracts (data not shown). These results argued that in the *dsbA*⁻ background, P pilus assembly was impaired because of a defect in chaperone–subunit complex formation.

Disulfide Bond Formation in PapG and Preassembly Complex Formation Occur Very Fast in a *dsbA*⁺ Background. The kinetics of disulfide bond formation in PapG in a *dsbA*⁻ or *dsbA*⁺ background was studied by a pulse–chase analysis after induction of the expression of PapD and PapG in JCB570/pJP2 (*dsbA*⁺*papDpapG*) or JCB571/pJP2 (*dsbA*⁻*papDpapG*). PapG was immunoprecipitated from periplasmic extracts of cells taken at various times during the chase by using anti-tip fibrillum antiserum that recognizes PapG but not PapD and the proteins were analyzed by nonreducing SDS/PAGE to distinguish the reduced and oxidized forms of PapG. In *dsbA*⁺ cells, PapG was already oxidized during the labeling period and it was associated with PapD as shown by the presence of a radiolabeled PapD band that coimmunoprecipitated with PapG (Fig. 2). The increase in the amount of oxidized PapG over time did not correlate with the decrease of the faint band of reduced PapG. This suggested that disulfide bond formation was extremely fast and occurred during or immediately after

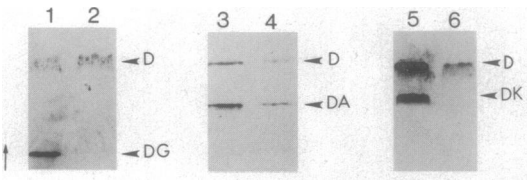

FIG. 1. Preassembly chaperone–subunit complexes in *dsbA*⁺ and *dsbA*⁻ strains. Periplasmic extracts of *dsbA*⁺ (lanes 1, 3, and 5) and *dsbA*⁻ (lanes 2, 4, and 6) expressing PapD and PapG (lanes 1 and 2), PapD and PapA (lanes 3 and 4), or PapD and PapK (lanes 5 and 6) were analyzed by acidic native PAGE and Western blotting developed using anti-PapD–PapG (lanes 1 and 2) or anti-PapD (lanes 3–6) antiserum. Arrow indicates the direction of migration. PapD and PapD–subunit complexes are indicated.

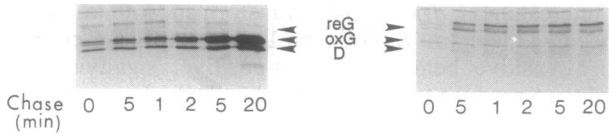

FIG. 2. Kinetics of disulfide bond formation in PapG in *dsbA*$^+$ and *dsbA*$^-$ strains. Autoradiogram showing radiolabeled PapG immunoprecipitated after various times of chase from periplasmic extracts of *dsbA*$^+$ cells (*Left*) or *dsbA*$^-$ cells (*Right*) analyzed by nonreducing SDS/12.5% PAGE. PapD–PapG complex was run in parallel under nonreducing or reducing conditions and detected by Coomassie staining to identify the corresponding radiolabeled bands. reG, reduced PapG; oxG, oxidized PapG; D, PapD.

the translocation of PapG through the cytoplasmic membrane. In *dsbA*$^-$ cells, a significant band of reduced PapG was detected that faded after 20 min (Fig. 2). A faint band of oxidized PapG was also detected that did not increase over time. Very little PapD was coimmunoprecipitated with PapG in the *dsbA*$^-$ strain. These observations suggested that the adhesin did not form its disulfide bonds efficiently in the absence of DsbA and that reduced PapG did not associate efficiently with PapD. The possibility that PapD was not functional in *dsbA*$^-$ cells and was therefore unable to associate with PapG was tested as described below. The addition of 5 mM oxidized glutathione to the culture medium of the *dsbA*$^-$ strain did not increase the amount of oxidized PapG or the amount of PapD which coimmunoprecipitated with the PapG (data not shown).

PapD Is Mostly Misfolded in the Absence of DsbA. The role of DsbA in catalyzing the formation of the disulfide bond in PapD was investigated. A kinetic analysis similar to that described above was performed with JCB570 (*dsbA*$^+$) or JCB571 (*dsbA*$^-$) cells expressing PapD from pHJ6. Due to the proximity of the two cysteines in the sequence (amino acids 207 and 212) and in the tertiary structure of PapD (ref. 19; Fig. 3*A*), the pulse-labeled periplasmic extracts were analyzed by native PAGE at pH 6.5 to distinguish oxidized (native) PapD from nonnative species. In *dsbA*$^+$ periplasmic extracts, a prominent labeled PapD band migrated to the same position as native purified PapD. In contrast, only a faint native PapD band that did not significantly increase over time was detected in *dsbA*$^-$ extracts (data not shown).

The folded state of the PapD species in the periplasm was investigated by determining the kinetics by which PapD acquired a protease-resistant conformation in *dsbA*$^+$ compared with *dsbA*$^-$ cells. Pulse-labeled periplasmic extracts such as those described above were subjected to limited proteolysis by trypsin. PapD and its fragments were immunoprecipitated and analyzed by SDS/PAGE. Trypsin cleaves native PapD in the F1–G1 loop, yielding domain 1 and domain 2 fragments (20). In the *dsbA*$^+$ strain the proteolytic fragments were detected in stoichiometric abundance relative to the undigested band, but in the *dbsA*$^-$ strain significantly smaller amounts of PapD were present that were almost completely digested by trypsin (Fig. 3*B*). This suggested that in the absence of DsbA only a small fraction of PapD folded into the native protease-resistant conformation, indicating that the formation of the disulfide bond may be an early critical event in the folding of PapD.

The Nonconserved Disulfide Bond of PapD Is Indispensable for P Pilus Assembly. The disulfide bond in PapD is absent from other members of the pilus chaperone family (2). Site-directed mutagenesis was performed to replace Cys^{207} and Cys^{212} with serines (pFJ34) or Cys^{212} with a nonsense codon, truncating PapD by 7 amino acids (pFJ33). The mutant chaperones were tested for their ability to complement a *papD*$^-$ operon expressed from pPAP37 in *dsbA*$^+$ strains. HB101 or ORN103 cells expressing mutant PapD from either pFJ34 or pFJ33 were hemagglutination-negative (titers $\leq$ 2) compared with cells expressing wild-type PapD from pLS101 (titers of 64 and 32, respectively) and were nonpiliated as detected by anti-pilus antiserum in an ELISA (titers < 1), compared with titers of $\approx$500 and $\approx$1000 for HB101 and ORN103 expressing wild-type PapD, respectively.

The ability of the mutant chaperones to form complexes with PapA and PapG was investigated by Western blotting of acidic native polyacrylamide gels of periplasmic extracts obtained from cells coexpressing wild-type PapD from pLS101 or mutant PapDs from pFJ33 or pFJ34 with either PapA or PapG from a compatible plasmid (pRS2A or pLS201, respectively). PapD–PapA and PapD–PapG chaperone–

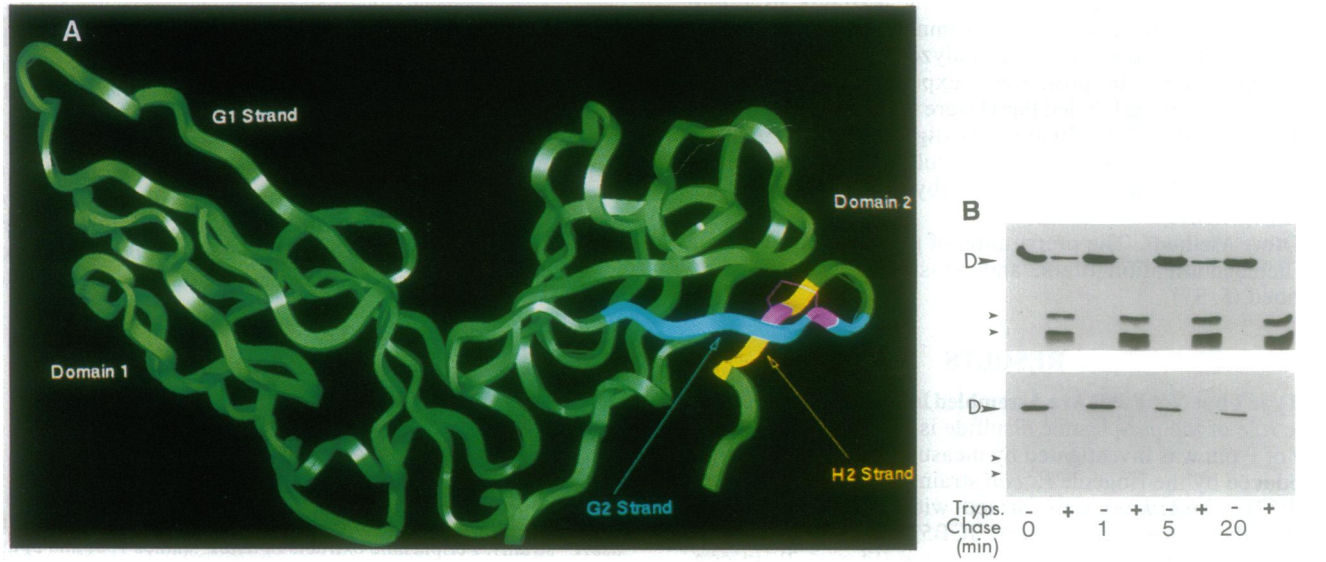

FIG. 3. Stability of PapD in wild-type and *dsbA*$^-$ strains. (*A*) Ribbon model of the three-dimensional structure of PapD. Strands G2 and H2 are in blue and yellow, respectively, with the disulfide-bonded cysteines in pink. Strand G1 was shown to be a major binding site for the subunits on PapD (20). (*B*) Autoradiogram showing pulse-labeled PapD from *dsbA*$^+$ (*Upper*) or *dsbA*$^-$ (*Lower*) periplasmic extracts incubated without (−) or with (+) trypsin (100 μg/ml) for 20 min at 37°C, immunoprecipitated with an anti-PapD antiserum, and analyzed by SDS/15% PAGE. Arrowheads indicate full-size PapD (D) and its tryptic fragments.

subunit complexes were detected in periplasmic extracts expressing wild-type PapD but not in the mutants. In addition, the mutant chaperones were present in very small amounts compared with wild-type PapD (data not shown). Limited tryptic digestions of pulse-labeled mutant present in periplasmic extracts of HB101/pFJ34 (C207S+C212S mutant PapD) compared with HB101/pLS101 (wild-type PapD) showed that in contrast to wild-type PapD, the tryptic fragments of the mutant PapD were in much lower than stoichiometric amounts relative to the undigested species (data not shown), arguing that the mutant PapD proteins did not fold into native conformations.

The functional role of the disulfide bond in PapD was tested in an *in vitro* subunit binding assay. Pure wild-type PapD was denatured and reduced and the sulfhydryl groups were alkylated with iodoacetic acid prior to renaturation (Fig. 4 *Inset*). Native or alkylated PapD was incubated with immobilized MBP–G140, and binding was detected in an ELISA using anti-PapD antiserum. Both PapD species bound equally well to the chimera (Fig. 4) and not to MBP alone and they formed complexes with MBP–G140 in solution, as detected by isoelectrofocusing (data not shown), indicating that the disulfide bond in PapD was not required for PapD to achieve a subunit binding conformation. In addition, the fluorescence emission spectra of urea-denatured reduced PapD diluted in a stepwise fashion demonstrated that *in vitro* refolding was achieved in the absence of disulfide bond formation (data not shown).

Subunit Disulfide Bond Formation *in Vivo*. It was difficult to determine whether disulfide bond formation in the subunits preceded preassembly complex formation, since a functional chaperone was not available in the *dsbA*$^-$ background. Therefore a pulse–chase experiment was performed with JCB570 (*dsbA*$^+$) or JCB571 (*dsbA*$^-$) cells that expressed PapG alone from pHJ8. PapG from periplasmic extracts of cell aliquots taken at various times of chase was immunoprecipitated with an anti-PapD–PapG antiserum. In the *dsbA*$^+$ background, a significant amount of oxidized PapG appeared after 1 min and somewhat decreased over time (Fig. 5). Some protein bands characteristic of limited *in vivo* proteolytic degradation of PapG (5) increased between 5 and 20 min. *dsbA*$^-$ periplasmic extracts did not contain significant amounts of PapG, and the characteristic products of limited proteolysis did not appear, indicating a more complete proteolytic degradation. Therefore it seems that DsbA catalyzes

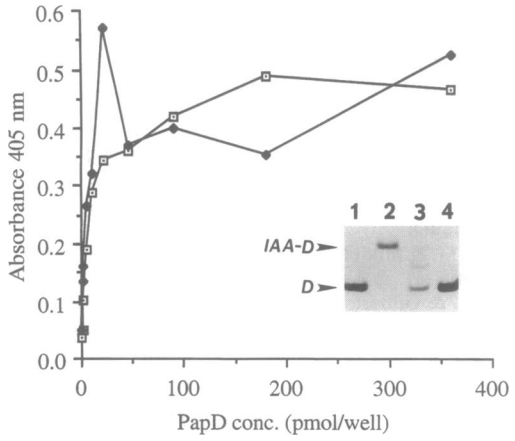

FIG. 4. *In vitro* function and folding of PapD. Curve showing the binding of native (▫) or alkylated (◆) PapD to immobilized chimeric protein MBP–G140 quantitated by ELISA using anti-PapD antiserum. (*Inset*) Coomassie-stained native polyacrylamide gel (pH 6.5) showing native PapD (lane 1) or PapD after various treatments: denatured, reduced, alkylated, and renatured PapD (lane 2); same as lane 2 except that the alkylating agent (lane 3) or the reducing agent (lane 4) was omitted. IAA-D, alkylated PapD; D, native PapD.

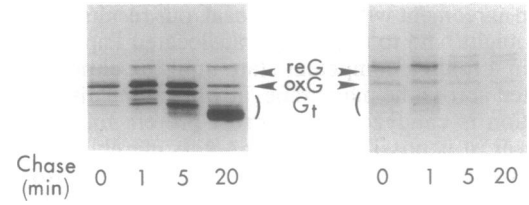

FIG. 5. Disulfide bond formation in PapG. Autoradiogram showing radiolabeled PapG immunoprecipitated after various times of chase from periplasmic extracts of *dsbA*$^+$ (*Left*) or *dsbA*$^-$ (*Right*) cells analyzed by nonreducing SDS/12.5% PAGE. reG, reduced PapG; oxG, oxidized PapG; G_t, truncates of PapG. The positions of reduced and oxidized PapG were determined as in Fig. 2.

the formation of disulfide bonds of PapG in the absence of PapD. If oxidized PapG does not interact with PapD it is subject to limited proteolytic degradation, whereas reduced PapG is more thoroughly degraded.

DISCUSSION

The *E. coli* periplasmic disulfide isomerase DsbA was required for P pilus assembly. The PapD chaperone was unable to fold into a native conformation able to bind subunits *in vivo* and mediate pilus assembly in the absence of DsbA. In contrast, denatured reduced PapD was able to refold and achieve a subunit binding conformation *in vitro* in the absence of disulfide bond formation. The discrepancy between *in vivo* and *in vitro* folding of PapD in the absence of its intramolecular disulfide bond may be due to a more stringent need to control folding (prevent misfolding) of the proteins *in vivo* as they emerge into the periplasmic space from the cytoplasmic membrane. One possibility is that DsbA has both oxidant and chaperone-like functions. Disulfide bond formation in pilus subunits was also mediated by DsbA even in the absence of PapD. However, the ability of pilus subunits to achieve protease-resistant conformations *in vivo* depended on PapD. These results argue that the productive folding pathway of subunits involves critical interactions with both DsbA and the PapD chaperone.

PapD possesses a pair of cysteines spaced only 5 amino acids apart that form an intrasheet disulfide bond that is unusual for an immunoglobulin-like protein. The disulfide bond occurs between the H2 and G2 β-strands in the CD4-like domain 2 (2, 19) as determined by x-ray crystallography. The H2 β-strand is not present in most PapD-like chaperones. The conversion of PapD's cysteines into serines by site-directed mutagenesis resulted in misfolded and nonfunctional PapD *in vivo*, mimicking the effects of the *dsbA*$^-$ mutation. Paradoxically, disulfide bond formation was not required for PapD folding or for its recognition function *in vitro*. *In vivo*, the translocation of PapD across the cytoplasmic membrane prior to folding may invoke the need for a chaperone-like activity possibly provided by DsbA.

DsbA has been implicated in accelerating disulfide bond formation in various secretory proteins such as OmpA, Bla, and PhoA (12, 13), and in the absence of DsbA defects in the assembly of oligomeric structures such as *E. coli* heat-labile enterotoxin (21), F pili (12), and cholera toxin and Tcp pili (22) have been reported. DsbA was proposed to act essentially as a donor of redox equivalents, an oxidant that needs to be regenerated (23, 24). In the presence of DsbA, PhoA acquired protease resistance following disulfide bond formation, indicative of a near-native conformation. In the absence of DsbA, PhoA was partially degraded *in vivo* (12, 13). The tertiary structure of DsbA shows the presence of an extensive hydrophobic surface close to its active site which has been suggested to bind partially folded polypeptides in a chaperone-like fashion, before the oxidation of cysteine residues

(25). In agreement with this proposal, our results suggest that DsbA might bind to nascently translocated PapD, maintaining it in a folding-competent state until the entire protein is exported across the membrane, at which time disulfide bond formation is catalyzed and correct protein folding ensues. However, it is possible that DsbA acts merely by oxidizing PapD when the cysteines emerge from the cytoplasmic membrane and that the formation of the disulfide bond is a critical event for the *in vivo* folding of PapD.

A chaperone role for DsbA in addition to its oxidoreductase function was already alluded to by Peek and Taylor (22) and Yu *et al.* (21). Other oxidoreductase proteins with a thioredoxin motif have also been suggested to have chaperone-like functions. Thioredoxin is absolutely required for filamentous phage f1 assembly, but not via its oxidoreductase activity, since its active-site cysteines can be mutated (26). Protein disulfide-isomerase (PDI) of the eukaryotic endoplasmic reticulum is essential in yeast even though active-site cysteine mutants are viable (27), and it was shown to have both catalytic and chaperone activities *in vitro* and to bind to misfolded and denatured proteins (28–30). The high concentrations of PDI and DsbA in their respective compartments, their rather low intrinsic catalytic activities, their peptide-binding activity, and the participation of PDI in several enzymatic complexes in which recognition of a nascent polypeptide chain is needed are all consistent with a chaperone activity (27, 31).

DsbA also acts on the pilus subunits. The rapid acquisition of disulfide bonds in PapG even in the absence of PapD suggested that oxidation precedes chaperone–subunit complex formation. In the absence of PapD, oxidized PapG was subject to a more limited proteolytic degradation than reduced PapG, suggesting that oxidized PapG achieves a more compact conformation or that the interaction with DsbA somewhat protects it. In a *dsbA*$^-$*papD*$^-$ background, PapG was completely degraded very rapidly. Related type 1 pili, whose chaperone (FimC) has no cysteines, were assembled, albeit less efficiently, in a *dsbA*$^-$ background. The reduced amount of type 1 pili assembled in a *dsbA*$^-$ background is probably due to the function of DsbA in mediating disulfide bond formation in the pilus subunits. Possible reasons for the failure of the *dsbA*$^-$ mutation to completely abolish type 1 pilus assembly include the following. The type 1 chaperone, FimC, lacks cysteine and was stable in the *dsbA*$^-$ cells (data not shown), suggesting that its function does not depend on DsbA. In addition, it is possible that in the absence of DsbA, oxidation of pilus subunits can proceed spontaneously or by an independent disulfide periplasmic oxidase such as DsbC, which can functionally substitute for DsbA (32).

It is possible that subunit folding in the periplasmic space is a sequential process involving at least two chaperones: DsbA binds the subunit during its translocation across the cytoplasmic membrane and, after catalyzing the formation of the subunit disulfide bond(s), it passes it onto PapD. Whether folding is completed prior to the interaction with PapD or on the PapD platform remains to be determined. Circular-dichroism spectra of pili suggest that PapA has predominantly a β-sheet structure (R.S. and C. Frieden, unpublished work). A peptide corresponding to the carboxyl terminus of PapG was shown to bind in the PapD cleft as a parallel β-strand zippering along the G1 β-strand of PapD, forming backbone hydrogen bonds and a pattern of alternating hydrophobic interactions with PapD (20). Does the subunit β-sheet form by extending from the G1 β-strand of PapD? A hydrophobic zipper mechanism has been suggested to nucleate the folding of all-β-sheet proteins (33, 34). A test of these models awaits *in vivo* and *in vitro* folding studies.

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